Baicalein attenuates oxidant stress in cardiomyocytes

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Shao, Zuo-Hui, Terry L. Vanden Hoek, Yimin Qin, Lance B. Becker, Paul T. Schumacker, Chang-Qing Li, Lucy Dey, Eugene Barth, Howard Halpern, Gerald M. Rosen, and Chun-Su Yuan. Baicalein attenuates oxidant stress in cardiomyocytes. Am J Physiol Heart Circ Physiol 282: H999–H1006, 2002; 10.1152/ajpheart.00163.2001.—Flavonoids within Scutellaria baicalensis may be potent antioxidants on the basis of our studies of S. baicalensis extract. To further this work, we studied the antioxidative effects of baicalein, a flavonoid component of S. baicalensis, in a chick cardiomyocyte model of reactive oxygen species (ROS) generation during hypoxia, simulated ischemia-reperfusion, or mitochondrial complex III inhibition with antimycin A. Oxidant stress was measured by oxidation of the intracellular probes 2′,7′-dichlorofluorescein diacetate and dihydroethidium. Via-,7-

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reactive oxygen species (ROS) generation during hypoxia, simulated ischemia-reperfusion, or mitochondrial complex III inhibition with antimycin A. Oxidant stress was measured by oxidation of the intracellular probes 2′,7′-dichlorofluorescein diacetate and dihydroethidium. Viable cell was assessed by propidium iodide uptake. Baicalein attenuated oxidant stress during all conditions studied and was present in minutes of treatment. For example, baicalein given only at reperfusion dose dependently attenuated the ROS burst at 5 min after 1 h of simulated ischemia. It also decreased subsequent cell death at 3 h of reperfusion from 52.3 ± 2.5% in untreated cells to 29.4 ± 3.0% (with return of contractions; P < 0.001). In vitro studies using electron paramagnetic resonance spectroscopy with the spin trap 5-methoxy carbonyl-5-methyl-1-pyrroline-N-oxide revealed that baicalein scavenges superoxide but does not mimic the effects of superoxide dismutase. We conclude that baicalein can scavenge ROS generation in cardiomyocytes and that it protects against cell death in an ischemia-reperfusion model when given only at reperfusion.

Scutellaria baicalensis; ischemia; reactive oxygen species; 2′,7′-dichlorofluorescein diacetate; antimycin A; dihydroethidium; mitochondria

Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) is one of the major flavonoids of Scutellaria baicalensis, an herb used in Chinese and Japanese medical applications. Among its biological activities, baicalein has been reported to exhibit antioxidant effects (9, 15, 20, 41). In this regard, it has been reported to scavenge reactive oxygen species (ROS), including superoxide (O2•−), H2O2, and hydroxyl radicals (14). Baicalein has also been shown to strongly inhibit iron-dependent lipid peroxidation in microsomes (9) and mitochondria (26). In glomerular mesangial cells, damage induced by exogenous H2O2 treatment was inhibited by baicalein (32). Although the specific mechanism of protection was not known, baicalein may be protective against oxidant injury by virtue of its ability to function as an iron chelator (13). By inhibiting the Fe2+-catalyzed Fenton reaction, baicalein may decrease the generation of hydroxyl radicals in the presence of H2O2 (1). Using in vitro methods, Gao et al. (10) reported that baicalein prevented human dermal fibroblast cell damage by ROS and was more effective than the iron chelator deferoxamine, hydroxyl radical scavengers including dimethyl sulfoxide and ethanol, the lipid peroxidase quenching agent α-tocopherol (vitamin E), and the xanthine oxidase inhibitor allopurinol. Collectively, these studies support the conclusion that baicalein exhibits antioxidant properties, although the specific mechanism of action is not fully understood. In addition, antioxidant studies of such in vitro or exogenous oxidant models may not predict success in treating endogenous intracellular oxidant stress.

There is evidence that intracellular ROS generation may contribute to the pathogenesis of cellular injury during ischemia and reperfusion in a number of tissues (12, 22). ROS may interact degeneratively with cellular components, including nucleic acids, proteins, and lipids, to compromise structure and function (19). Moreover, the resulting functional defects may depend on the specific microdomains where the oxidants are generated. Although baicalein has been shown to confer protection against exogenously applied oxidants, its ability to attenuate cell injury has not been demonstrated in a pathophysiological model where the ox-
dants generated within the cell contribute to the cellular dysfunction. Reactive species, including $\text{O}_2^-$, $\text{H}_2\text{O}_2$, and hydroxyl radicals, have been shown to contribute to myocardial ischemia-reperfusion injury (7, 27). A similar role for ROS has been demonstrated in the contractile dysfunction and cell death observed during simulated ischemia-reperfusion and mitochondrial inhibition in a cardiomyocyte model (35–37). The observation that antioxidants confer significant protection in cultured cardiomyocytes suggests that such a model is appropriate for testing whether baikalein protects against oxidant injury. In addition, since many antioxidants previously found to be effective in these settings require preincubation (36) or must be given as a cocktail (39), this model allows testing of whether a single antioxidant administered at the time of oxidant generation can confer protection without preincubation. Such antioxidants could prove useful in modifying injury where other antioxidants have failed because of difficulties of intracellular access (27). Our previous study demonstrated that a water extract of Scutellaria baicalensis could attenuate oxidant stress even when given just at the start of conditions that increase endogenous oxidant generation (32). The present study was designed to extend those findings by examining the antioxidant and cardioprotective properties of one of the principal flavonoids in that extract.

METHODS

Diethylenetriaminepentaacetic acid (DTPA), xanthine, xanthine oxidase, catalase, superoxide dismutase (SOD), baikalein (98% purity), and antimycin A were purchased from Sigma (St. Louis, MO), 5-hydroxydecanoate-Na (5-HD) from Biomol (Plymouth Meeting, PA), and diazepam-binding inhibitor from Calbiochem (San Diego, CA). The spin trap 5-methoxycarbonyl-5-methyl-1-pyrroline-N-oxide (MMPO) was synthesized on the basis of methods described previously (5, 33). The structure of baikalein and the reaction of MMPO with $\text{O}_2^-$ are shown in Fig. 1.

Cardiomyocyte System

Embryonic ventricular cardiomyocytes were prepared as described previously (35). Briefly, heart ventricles from 10-day-old chick embryos were removed, minced, enzymatically dispersed with 0.025% trypsin (GIBCO, New York, NY), and centrifuged to yield $4 \times 10^5$ cells/embryo. Cells ($0.7 \times 10^6$) were pipetted onto glass coverslips, incubated, and grown into contractile layers. Synchronous contractions were seen by the third day in culture. Contamination by fibroblasts was reduced by preplating, and myocyte phenotype was confirmed using antymyosin heavy chain monoclonal antibodies (CCM-52). Experiments were performed with 3- to 5-day cardiac cell culture, at which point viability was $>99\%$.

Perfusion System

Coverslips with synchronously contracting cells were placed inside a Sykes-Moore flow-through chamber (1.2 ml volume; Bellco Glass, Vineland, NJ). The chamber and inflow tubing were maintained at 37°C. Flow rate (0.25 ml/min), pH, and $\text{P}_2\text{O}_2$ of the perfusate were controlled. Hypoxic conditions were verified with an optical method of phosphorescence quenching (Oxyspot, Medical Systems, Greenvale, NY). To minimize $\text{O}_2$ leaks, the perfusate was supplied to the chamber by stainless steel tubing to prevent diffusive entry of $\text{O}_2$ through the tube wall.

Perfusate Composition

Standard perfusate consisted of buffered salt solutions (BSS) with 100 Torr $\text{P}_2\text{O}_2$, 40 Torr $\text{P}_2\text{CO}_2$, pH 7.4, 4.0 meq $\text{K}^+$/l, and 5.6 mM glucose. Simulated ischemia consisted of BSS containing no glucose, with 20 mM 2-deoxyglucose added to inhibit glycolysis and 8 meq $\text{K}^+$/l. This was bubbled with 80% $\text{N}_2$-20% $\text{CO}_2$ to produce $<3$ Torr $\text{P}_2\text{O}_2$, 144 Torr $\text{P}_2\text{CO}_2$, and final pH 6.8. Hypoxic medium was bubbled with 95% $\text{N}_2$-5% $\text{CO}_2$ and without glucose. Reperfusion was carried out with standard BSS.

Video/Fluorescent Microscopy

Cells were imaged with an Olympus IMT-2 inverted phase/epifluorescent microscope equipped with Hoffman Modulation optics to accentuate the surface topology of the cells. This facilitated detection of contractile movement in the confluent layer of cells. Cell contractions were observed as described previously (32, 36). The criteria for a return of contraction were met if contractions were observed throughout the cell field after 3 h of reperfusion. A single field of cells was monitored for contractions throughout each experiment. Phase-contract images were recorded for contraction analysis with a charge-coupled device camera. Fluorescence was measured using a cooled slow-scanning personal computer-controlled camera (Hamamatsu, Hamamatsu City, Japan) coupled with Image-One software (Image Pro Plus) for quantification of changes in emission fluorescence.

Viability Assay

Cell viability was quantified over time using the nuclear stain propidium iodide (PI, 5 $\mu$M; Molecular Probes, Eugene, OR), an exclusion fluorescent dye that binds to chromatin on loss of membrane integrity. This method is similar in principle to trypan blue staining and has been reported to predict the transition from reversible to irreversible cell injury in cultured cardiomyocytes (4). PI is not toxic to cells over a course of 8 h, permitting its addition to the perfusate.
throughout the experiment. At the end of each experiment using PI, all nuclei in a field of ~500 cells were stained by permeabilization with 300 μM digitonin. Percent loss of viability (i.e., cell death) over time was expressed relative to the maximal value seen after digitonin exposure (100%).

**Measurement of Intracellular ROS Generation**

Intracellular oxidant stress was monitored by measuring changes in fluorescence resulting from intracellular probe oxidation. Dihydroethidium (DHE, 2 μM; Molecular Probes) enters the cell and can be oxidized by ROS, including O$_2^*$ and/or hydroxyl radical, to yield fluorescent ethidium (Eth). Eth binds to DNA (Eth-DNA), further amplifying its fluorescence. Thus increases in DHE oxidation to Eth-DNA (i.e., increases in Eth-DNA fluorescence) are suggestive of O$_2^*$ generation (36). The probe 2’,7’-dichlorofluorescein diacetate (DCFH-DA, 5 μM; Molecular Probes) enters the cell, and the acetate group on DCFH-DA is cleaved by cellular esterases, trapping the nonfluorescent 2’,7’-dichloro fluorescin (DCFH) inside. Subsequent oxidation by ROS, particularly H$_2$O$_2$ and hydroxyl radical, yields the fluorescent product dichlorofluorescein (DCF) (28). Thus increases in DCFH oxidation to DCF (i.e., increases in DCF fluorescence) suggest H$_2$O$_2$ or hydroxyl radical generation (36).

**Measurement of In Vitro ROS Generation**

Spin trapping of O$_2^*$ by 110 mM MMPO was achieved as follows. All experiments were carried out in phosphate-buffered (50 mM) saline (PBS) containing 1 mM DTPA to chelate any trace redox active metal ions that contaminate PBS. Xanthine (0.40 mM) was dissolved in PBS. Catalase (300 U/ml) was added to the reaction mixture to eliminate H$_2$O$_2$ produced by the self-dismutation of O$_2^*$. The reaction was initiated by addition of xanthine oxidase (0.04 U/ml) to the above mixture. This resulted in an initial rate of O$_2^*$ production of ~10 μM/min. The reaction mixture was then loaded into a quartz aqueous flat cell (Wilmac) and installed into a horizontally disposed cavity (TM011) of the electron paramagnetic resonance (EPR) spectrometer (model E-12, Varian Associates, Palo Alto, CA). The ability of baicalein to scavenge O$_2^*$ was also assessed with 10 μM DCFH-DA using a xanthine (0.4 μM)xanthine oxidase (0.02 U/ml) system to generate O$_2^*$. In the presence of SOD (200 U/ml), all O$_2^*$ would be expected to quickly become H$_2$O$_2$ and oxidize DCFH to DCF. Baicalein (10 μM) was added to the system to measure the changes of DCF fluorescence using a fluorescence spectrophotometer (excitation 488 nm/emission 529 nm).

**Conditions Used to Generate ROS and Induce Oxidant Injury**

**Brief hypoxia protocol.** Cardiomyocytes were preincubated for 45 min with 5 μM DCFH-DA, perfused for 15 min with standard BSS, and then exposed to hypoxia for 10 min and normoxia for 10 min. Baicalein was added to the perfusate only during the hypoxia phase.

**Simulated ischemia protocol.** Cells were loaded with 2 μM DHE, equilibrated for 30 min with standard BSS, and then exposed to 1 h of simulated ischemia. Baicalein at various doses was given during the ischemia phase.

**Simulated ischemia-reperfusion.** Cells were loaded with 5 μM DCFH-DA, equilibrated for 30 min with normoxia, and then exposed to 1 h of ischemia, followed by 30 min of reperfusion. Baicalein was added only during reperfusion. Cells were loaded with 5 μM PI, equilibrated for 30 min with normoxia, and exposed to 1 h of ischemia and 3 h of reperfusion and then to 300 μM digitonin for 1 h. Baicalein was added only during reperfusion.

**Mitochondrial inhibition.** Cells were loaded with 10 μM DCFH-DA or 5 μM PI and exposed to 100 μM antimycin A alone or with baicalein for 2 h in multiple culture dishes.

**Data Analysis**

Data were collected, and simple descriptive analyses were performed. An individual experiment (n) was the result of observations of a single field of ~500 cells on a coverslip. Replicates were performed on separate coverslips. Values are means ± SE. For test of significance, analysis of variance and two-tailed unpaired t-test were performed, with P < 0.05 considered to be significant.

**RESULTS**

**Effect of Baicalein on Oxidant Stress During Brief Hypoxia**

Oxidant stress was assessed by measuring oxidation of DCFH to DCF. As shown in Fig. 2, brief hypoxia caused a rapid and significant increase in DCF fluorescence (measured in arbitrary units) from 0.71 ± 0.11 (SE) at baseline to 1.70 ± 0.07 (n = 6, P < 0.01) at 10 min of hypoxia. Baicalein, at 25, 50, 100, or 200 μM, given at the start of hypoxia caused a dose-dependent attenuation in DCF fluorescence to 1.57 ± 0.09 (n = 6, P > 0.01), 1.33 ± 0.05 (n = 6, P < 0.01), 0.84 ± 0.10 (n = 6, P < 0.001), and 0.62 ± 0.05 (n = 6, P < 0.001), respectively. These findings suggest that baicalein caused a concentration-dependent attenuation of H$_2$O$_2$ and hydroxyl radicals during transient hypoxia.
**Effect of Baicalein on Oxidant Stress During Simulated Ischemia**

Oxidation of DHE to Eth-DNA was used to assess $O_2^\cdot$- and hydroxyl radical generation during simulated ischemia. A significant increase in Eth-DNA fluorescence was seen during 1 h of ischemia (Fig. 3). However, 5 or 10 μM baicalein attenuated Eth-DNA fluorescence from 100% in untreated ischemic cells to 66.7 ± 6.2% (n = 5, P < 0.01) and 48.2 ± 4.6% (n = 5, P < 0.001), respectively. This suggests that baicalein can attenuate oxidant stress due to $O_2^\cdot$ during simulated ischemia.

**Effect of Baicalein on Oxidant Stress, Cell Viability, and Contractile Function During Ischemia and Reperfusion**

A rapid burst of DCF fluorescence was observed at 30 min of reperfusion after 1 h of ischemia (Fig. 4). In cells treated with 25 or 100 μM baicalein only during reperfusion, DCF fluorescence was attenuated from 2.88 ± 0.21 in untreated ischemic cells (n = 5) to 2.11 ± 0.09 (n = 5, P < 0.01). In cells treated with 100 μM baicalein, DCF fluorescence was further attenuated to 1.43 ± 0.07 (n = 5, P < 0.001). These findings suggest that baicalein given only at reperfusion attenuated oxidant stress during reperfusion in a dose-dependent fashion.

As reported previously, cell death in this model of simulated ischemia-reperfusion occurred primarily during the reperfusion phase, whereas minimal cell death was seen during the ischemia phase (35). Significant cell death again was evident during 3 h of reperfusion after 1 h of ischemia. In cells treated with 50 μM baicalein given only during reperfusion, PI uptake decreased from 52.3 ± 2.5% in untreated ischemic cells (n = 6) to 29.4 ± 3.0% (n = 3, P < 0.001; Fig. 5). Contractile activity returned after reperfusion (3 of 3 experiments in treated cells), whereas there was no recovery of contraction in untreated cells (0 of 6 experiments in controls). Thus baicalein significantly reduced cell death and enhanced the return of contraction.

**Mechanism of Baicalein Effect on Reperfusion ROS**

ATP-sensitive K+ (KATP) channels have been implicated in protection of cardiomyocytes against ischemia-reperfusion injury. Indeed, our own recent work suggests that KATP channel opening just at reperfusion can attenuate oxidant generation and confer significant cardioprotection (39). To determine whether KATP channels were responsible for the protection afforded by baicalein, a KATP channel inhibitor, 5-HD, was given during equilibration and ischemia-reperfusion. DCF fluorescence was attenuated from 2.88 ± 0.21 in untreated ischemic cells (n = 5) to 2.03 ± 0.06 (n = 3, P < 0.01) in baicalein (25 μM)-treated cells also given 500 μM 5-HD. This response was not different from...
that seen with baicalein alone (from 2.88 ± 0.21 in untreated cells to 2.11 ± 0.09, n = 5, P < 0.01; Fig. 6). Thus inhibition of the K<sub>ATP</sub> channel did not abolish the protective effect of baicalein.

Baicalein has been reported to have an affinity for the benzodiazepine binding site of GABA<sub>A</sub> receptors (23). Interestingly, the benzodiazepine receptors in peripheral tissues such as adrenals, kidney, and heart (2) enhance mitochondrial processing of human manganese-dependent SOD (Mn-SOD) precursor protein. Wright et al. (40) suggested a possible redox-related mechanism of mitochondrial protein import that may lead to less efficient precursor protein uptake by mitochondria under severely oxidizing conditions. In our study, we tested whether the antioxidant effect of baicalein is related to this benzodiazepine binding site of peripheral benzodiazepine receptors. When 25 μM baicalein was given during the ischemia and reperfusion phases, DCF fluorescence was attenuated from 2.50 ± 0.13 to 1.76 ± 0.10 (n = 3, P < 0.01). In cells treated with 1 μM diazepam-binding inhibitor and 25 μM baicalein, DCF fluorescence was also attenuated to 1.81 ± 0.13 (n = 3, P < 0.01; Fig. 7). Thus inhibition of the benzodiazepine receptor did not abolish the protective effect of baicalein.

**Effect of Baicalein on Oxidant Stress and Cell Viability During Mitochondrial Electron Transport Inhibition**

Antimycin A inhibits mitochondrial electron transport through complex III at a site that enhances generation of O<sub>2</sub>·⁻ (6). Figure 8 shows that antimycin A increased DCF fluorescence from 232 ± 27 in controls (n = 10) to 1,883 ± 105 at 2 h of exposure as expected. By contrast, DCF fluorescence in cells exposed to 100 μM antimycin A and treated with 10 or 50 μM baicalein was attenuated to 997 ± 75 (n = 10, P < 0.01) or 822 ± 65 (n = 10, P < 0.001), respectively. These findings indicate that baicalein caused a concentration-dependent attenuation of oxidant stress induced by mitochondrial electron transport inhibition with antimycin A.

Figure 9 shows that cell death was significantly increased at the end of 2 h of antimycin A exposure from 4.8 ± 1.5% in controls (n = 10) to 51.1 ± 3.2% (n = 10). In cells exposed to 100 μM antimycin A and treated with 10 or 50 μM baicalein, cell death was decreased to 25.5 ± 2.3% (n = 10, P < 0.01) and 20.1 ± 1.8% (n = 10, P < 0.001), respectively. Thus baicalein decreased cell death during mitochondrial electron transport inhibition with antimycin A.

**ROS Scavenging by Baicalein**

Fluorophores such as DCFH and DHE can potentially be oxidized by multiple ROS, so they lack the
speciflcity required to implicate a particular oxidant. Accordingly, studies were carried out using the spin trap MMPO, which reacts with O$_2^-$ to create an adduct (MMPO-OOH) with a unique EPR spectrum. Superoxide was generated in vitro using a xanthine/xanthine oxidase system. In the absence of baicalein, the MMPO-OOH levels increased for the first 15 min, then decreased toward zero as the rate of O$_2^-$ generation declined and the MMPO-OOH adduct decayed spontaneously (Fig. 10). Addition of SOD abolished the increase in MMPO-OOH, confirming that the EPR spectrum reflected the spin trapping of O$_2^-$ . Addition of baicalein produced a concentration-dependent attenuation of the EPR spectrum of MMPO-OOH, suggesting that baicalein scavenged O$_2^-$.

With the use of in vitro studies with DCFH-D A in a xanthine/xanthine oxidase system, minimal oxidation of DCFH was observed in the absence of SOD, suggesting that DCFH oxidation by O$_2^-$ is minimal. Addition of SOD caused a significant increase in DCFH oxidation, suggesting that this probe is susceptible to H$_2$O$_2$. Addition of baicalein to the SOD-containing system caused a marked decrease in the oxidation of DCFH, suggesting that baicalein can scavenge O$_2^-$ and that it does not act as an SOD mimetic (Fig. 11).

**DISCUSSION**

_S. baicalensis_ is a widely used herb in traditional medical systems of China and Japan (17). The major constituents of _S. baicalensis_ are flavonoids, a group of polyhydroxy phenols (20). Flavonoids of _S. baicalensis_, which include baicalein, baicalin, wogonin, and skullcap flavones I and II, have been associated with antioxidant and other pharmacological effects. Among them, baicalein has attracted considerable attention, because it has a variety of interesting activities. As a polyphenol, which belongs to the flavone subgroup, it potentially has potent free radical scavenging and antioxidant effects because of its o-trihydroxy structure in the A ring (11). The antioxidant effectiveness of phenolic compounds may relate to their ability to enter cells and to orient in biomembranes (18). Flavonoids anchor to the polar heads of membrane phospholipids, forming reversible physicochemical complexes (29). The degree of glycosylation is one characteristic that affects various properties of some flavonoids, particularly their hydrophobicity (24). For example, the glycosidic group of rutin, a flavonol, makes it unable to penetrate model membranes (29). Baicalein, being free of sugar moieties, is more lipid soluble and may be able to penetrate membranes with greater ease. This lipophilic characteristic of baicalein may explain why its antioxidant effects could be seen within minutes of treatment against the various conditions under which the endogenous generation of ROS was increased.
Effect of Baicalein on Oxidant Stress During Moderate Hypoxia

We previously reported that 10 min of hypoxia in chick cardiomyocytes elicited an increase in mitochondrial ROS generation, as evidenced by oxidation of the intracellular fluorescent probe DCFH (38). These low levels of ROS appear to participate as signaling messengers. In the present study, increases in DCF oxidation during hypoxia were attenuated in a dose-dependent manner after addition of baicalein to the perfusate, suggesting that baicalein can attenuate oxidant signaling in cells. To rule out the alternative possibility that baicalein might interfere with DCFH oxidation measurements, EPR spectroscopy experiments were carried out using MMPO as a spin trap for O₂⁻ generated by xanthine/xanthine oxidase. The corresponding EPR spectrum of MMPO-OOH is unique for O₂⁻. The EPR spectroscopy data indicate that baicalein can scavenge O₂⁻. In other in vitro studies, DCFH oxidation by H₂O₂ was assessed in a xanthine/xanthine oxidase system. DCFH oxidation was dramatically increased in the presence of SOD, indicating that DCFH oxidation by H₂O₂ was greater than that by O₂⁻. In that system, baicalein attenuated the DCFH oxidation, suggesting that baicalein can scavenge O₂⁻ or H₂O₂ and that it does not act as an SOD mimetic.

Effect of Baicalein on Oxidant Stress During Ischemia

As reported previously (36, 37), an increase in the rate of DHE oxidation was seen during the ischemic phase in the present study. Our work has suggested that this oxidant stress is due to O₂⁻ generated from residual O₂ present during ischemia, since the DHE oxidation can be attenuated by exogenous SOD and is increased by SOD inhibition (3, 38). The attenuation of DHE oxidation seen during ischemia by baicalein further confirms that baicalein can act as a potent O₂⁻ scavenger.

Effect of Baicalein on Reperfusion Oxidants and Reperfusion Injury

Evidence from animal studies suggested that reperfusion of ischemic areas and the readmission of O₂ may cause further tissue damage (25). ROS, including O₂⁻, H₂O₂, and hydroxyl radicals, are responsible for reperfusion injury after myocardial ischemia (16, 42). Measures of free radical production have detected surges of ROS, particularly hydroxyl radical, during the first few seconds of reperfusion (42). In the present study, we observed a rapid (within 5 min) burst of DCF fluorescence at reperfusion that was reduced after treatment with baicalein. Also, cell death decreased and contractile activity returned in baicalein-treated cells. These observations indicate that baicalein confers significant protection in a model where oxidants are generated within cells during ischemia-reperfusion. Of the individual ROS scavengers evaluated in our system, baicalein has demonstrated the most effective protection against ischemia-reperfusion injury.

Effect of Baicalein on Oxidant Stress During Mitochondrial Electron Transport Chain Inhibition

Under physiological conditions, oxidant generation from the mitochondrial electron transport chain (ETC) is balanced by cellular antioxidant defenses (8). However, increases in O₂⁻ generation induced by ETC blockade with antimycin A (34) can create a lethal oxidant stress that overwhelms antioxidant defenses. When isolated cardiomyocytes were exposed to antimycin A, we observed an immediate increase in DCF fluorescence and an increase in cell death after 2 h. However, in cells exposed to antimycin A and treated with baicalein, the oxidant stress was attenuated as indicated by a decline in DCF fluorescence, and cell death also decreased in a dose-dependent manner. These findings support the conclusion that baicalein is protective under conditions where intracellular ROS generation is increased.

Mechanism of Baicalein Antioxidant Activity

Cellular antioxidants can act by inhibiting free radical formation, by directly scavenging radicals, or by enhancing cellular antioxidant mechanisms. Baicalein may protect by one or more of these mechanisms. In a previous study, we found that the mitochondrial Kₐ₅₆₉ channel modulates oxidant generation at the start of reperfusion (39). When the mitochondrial Kₐ₅₆₉ channel opener pinacidil was added at the start of reperfusion, it abrogated oxidant generation at reperfusion and reduced cell death. The mitochondrial Kₐ₅₆₉ channel inhibitor 5-HD (30) blocked this effect, which suggests that activation of this channel may regulate oxidant generation or antioxidant defenses in the cell. To test whether this channel is involved in the protective effects of baicalein, we tested whether 5-HD could abolish the antioxidant effects conferred by that flavonoid. DCF fluorescence increased during reperfusion after simulated ischemia in cardiomyocytes. However, attenuation of the reperfusion oxidant burst by baicalein was not blocked by 5-HD, indicating that the antioxidant action of baicalein is not mediated by activation of the mitochondrial Kₐ₅₆₉ channel.

Studies have also reported that the mitochondrial benzodiazepine receptor (mBzR) regulates multiple conductance channel activity. Moreover, mBzR agonists potentiate multiple conductance channel electrical activity (21) and enhance mitochondrial processing of Mn-SOD precursor protein (40). We therefore tested whether mBzR inhibition could abolish baicalein’s antioxidant effect. However, no effect of mBzR inhibition was detected, suggesting that this system is not involved.

In summary, our results show that baicalein attenuated oxidant stress in cardiomyocytes during hypoxia, ischemia, ischemia-reperfusion, and mitochondrial ETC inhibition. Antioxidant action is associated with increased cardiomyocyte survival and contractile function during ischemia-reperfusion. Our results also show that the antioxidant effect of baicalein is not associated with the mitochondrial Kₐ₅₆₉ channel or the
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